

Attorney Docket No.: 54704.8036.US03 (UMD-0072)  
Inventors: John Langenfeld  
Serial No.: 10/692,824  
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The following sections of the specification will replace the referenced sections of the specification.

Please replace paragraph 00144 at page 54 with the following paragraph:

--To perform RDA, mRNA was purified from the samples using ~~Oligo-dT~~ OLIGO DT columns (Pharmacia, Peapack, NJ) according to the manufacturer's instructions and cDNA was then obtained using the Pharmacia ~~Time-Saver~~ TIME SAVER cDNA synthesis kit also according to the manufacturer's instructions. cDNA was digested with Sau3A I endonuclease, R-linker ligated, and amplified by PCR. The R-linkers were removed and J-linkers ligated to the tester. The driver and tester cDNA were hybridized at 67°C for 20 hours (driver in excess 100:1) and the subtracted tester cDNA amplified by PCR. A second round of subtraction was performed using N-linkers (driver in excess 800,000:1). The amplified PCR products were cloned into blue script and sequenced using an ~~ABI-Prism~~ ABI PRISM 377 DNA sequencer. Known genes corresponding to the subtracted tumor cDNA were identified by a BLAST database search.--

Please replace paragraph 00151 at page 58 with the following paragraph:

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-Nude mice studies were conducted to determine the effect of BMP-2 and noggin on tumor growth and tumor vasculature.  $10^6$  A549 cells were injected subcutaneously into nude mice with ~~Affi-Blue~~ AFFI-BLUE agarose beads coated with albumin, recombinant human BMP-2, or recombinant mouse noggin. Both of these recombinant proteins were purchased from R&D Systems and were reconstituted in PBS with gelatin. Coating of ~~Affi-Blue~~ AFFI-BLUE agarose beads with BMP-2 and noggin has been described in the literature (Abe, E., et al., J. Bone Miner. Res. 15:663-673 (2000); Tucker, A.S., et al., Science 282:1136-1138 (1998); Zimmerman, L.B., et al., Cell 86:599-606 (1996)). In brief, 25 ug of ~~Affi-Blue~~ AFFI-BLUE agarose beads were incubated with 100 ug/ml albumin, recombinant human BMP-2, or recombinant noggin for 2 hours and then washed 3 times with PBS immediately prior to use. In separate experiments, the beads were not washed prior to injection. The coated beads were injected with the A549 cells into nude mice subcutaneously. To assess tumor growth after 12 or 19 days, the length, width, and depth of the tumors were measured in mm. To assess tumor vasculature, tissue including a tumor was harvested after six days. Gross observations of the tissue were made. In addition, the tissue was stained with anti-CD31 antibody, which recognizes endothelial cells. Vessels in

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five high power fields were counted by a person blinded to how the tumors were created.--

Please replace paragraph 00155 at page 60 with the following paragraph:

-To detect BMP-induced migration in a monolayer culture, recombinant human BMP-2 (R&D Systems, Minneapolis, MN) was coated to ~~Affi-Blue~~ AFFI-BLUE agarose beads (Bio Rad, Hercules, CA) as described in the literature. (Vaino, S. et al., Cell 75:45-58 (1993); Sloan, A.J. et al., Arch. Oral Biol. 44:149-156 (1999)) Briefly, 100 ml of the ~~Affi-Blue~~ AFFI-BLUE agarose beads were incubated with either 10 ml of recombinant BMP-2 reconstituted in PBS with gelatin (100 mg/ml) or PBS alone at 37°C for 2 hours, washed with PBS, and reconstituted with 40 ml of PBS. Glass cover slips were coated with serum free media containing BSA, fibronectin and collagen and 50,000 cells were plated per cover slip in serum free media. Two microliters of the ~~Affi-Blue~~ AFFI-BLUE agarose beads coated with recombinant BMP-2 or dilution buffer were placed in linear fashion next to the cover slips.--

Please replace paragraph 00157 at page 61 with the following paragraph:

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-Invasion was studied using transwell chambers coated with 100 ml of ~~Matrigel~~ MATRIGEL (Becton Dickinson). Fifty thousand cells were placed in the upper chamber and 300 ml of serum free media with 0 ng/ml, 10 ng/ml, 100 ng/ml, 500 ng/ml, or 1000 ng/ml recombinant BMP-2 placed in the lower wells. After 48 hours, the ~~Matrigel~~ MATRIGEL was removed and cells that had migrated through the filter were stained with Syto-16 intercalating dye and 5 high power fields counted using fluorescent microscopy.--

Please replace paragraph 00161 at page 63 with the following paragraph:

-To assess whether BMP-2 stimulates the migration of tumor cells growing in monolayer culture, it was examined whether A549 and H7249 cells growing on glass cover slips migrated toward ~~Affi-Blue~~ AFFI-BLUE beads coated with recombinant human BMP-2. Recombinant human BMP-2 (R&D Systems, Minneapolis, MN) was coated with ~~Affi-Blue~~ AFFI-BLUE agarose beads (bio Rad) as previously described. Briefly, 100  $\mu$ l of the ~~Affi-Blue~~ AFFI-BLUE agarose beads were incubated with 10  $\mu$ l of BMP-2 (100  $\mu$ g/ml) or PBS with 0.1% gelatin at 37°C for 2 hours. The beads were then washed with PBS and reconstituted with 40  $\mu$ l of PBS. Glass cover slips were coated with serum free media containing BSA, fibronectin and

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collagen and 50,000 cells were plated per cover slip. After allowing the cells to attach for 12 hours the cover slips were placed into a 6 well plate containing serum free media. Two microliters of the ~~Affi-Blue~~ AFFI-BLUE agarose beads coated with recombinant BMP-2 or dilution buffer were placed in linear fashion next to the cover slips. Five days later the cells, which migrated off the cover slips, were photographed.--

Please replace paragraph 00162 at page 64 with the following paragraph:

-The next issue was whether BMP-2 enhanced the invasiveness of tumor cells by determining whether recombinant BMP-2 stimulated the migration of A549 and H7249 cells through the extracellular tumor matrix, ~~Matrigel~~ MATRIGEL (Becton Dickinson, Bedford, MA). 100 µl of ~~Matrigel~~ MATRIGEL was placed in the upper well of an 8-micron transwell migration chamber. Fifty-thousand A549 or H7249 cells were placed in the upper chamber and 300 µl of LHC serum free media supplemented with recombinant human BMP-2 (1, 10, 100, or 500 ng/ml) or an equal volume of PBS with 0.1% gelatin was added to the lower well. After 48 hours, the ~~Matrigel~~ MATRIGEL the cells on the upper side of the filter were removed using a cotton swab. Cells

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that migrated through the filter were stained with Syto-16 nuclear dye and examined by fluorescent microscopy.--

Please replace paragraph 00165 at page 65 with the following paragraph:

-The m-RNA obtained from the driver and tester was size fractionated on a 1% agarose-formaldehyde gel in a MOPS (0.2M 3-N-morpholino-propanesulfonic acid/0.05M Na Acetate/0.01M EDTA) buffer. The m-RNA was transferred to a nitrocellulose membrane by capillary transfer. The m-RNA was cross-linked to the membrane using ultraviolet light. The subtracted BMP-2/4 cDNA was radiolabeled with P <sup>32</sup> using the ~~All-In-One Random Priming mix~~ ALL IN ONE RANDOM PRIMING MIX (Sigma Chemical, St. Louis, MO). The probe was denatured by boiling and incubated with the blots in ~~PerfectHyb-Plus~~ PERFECTHYB PLUS hybridization buffer (Sigma) for 12 hours. Membranes were washed in high stringent conditions and exposed to ~~Kodak~~ KODAK XAR film with an intensifying screen.--

Please replace paragraph 00166 at page 66 with the following paragraph:

-Total RNA was extracted from a patient derived tissue using ~~Trizol~~ TRIZOL (Gibco, Rockville, MD). First strand cDNA was

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synthesized using the ~~Advantage for PCR kit~~ ADVANTAGE FOR PCR KIT (Clontec, Palo Alto, CA) following the manufacturer's instructions. BMP-2 cDNA was amplified using primers (F)5'-cct gag cga gtt cga gtt g-3' [SEQ ID NO: 17], and (R)5'-cac tcg ttt ctg gta gtt c-3' [SEQ ID NO. 18] at 95°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes for 30 cycles. The expected size of the amplified BMP-2 was 230 base pairs. BMP-4 was amplified using primers (F)5'-tac ctg aga cgg gaa gaa a-3' [SEQ ID NO: 19] and (R)5'-cca gac tga agc cgg taa ag-3' [SEQ ID NO: 20] at 95°C for 1 minute, 56°C for 1 minute, 72°C for 2 minutes for 33 cycles. The expected size of the amplified BMP-4 was 211 base pairs. The amplified bands were gel purified and sequenced at the core UMDNJ-RWJMS sequencing facility using an ~~ABI-Prism~~ ABI PRISM 377 DNA sequencer.--

Please replace paragraph 00170 at page 68 with the following paragraph:

-To assess the effects of BMP-2 on tumor growth *in vivo*, A549 cells were coinjected subcutaneously into female NCJ athymic nude mice with recombinant BMP-2 or the BMP-2 antagonist noggin. Recombinant protein was delivered to the tumors using ~~Affi-Blue~~ AFFI-BLUE agarose beads as previously described. In brief, 25 µg of ~~Affi-Blue~~ AFFI-BLUE agarose beads were incubated with 20 µl of

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100 µg/ml of BSA, recombinant human BMP-2, or noggin for 2 hours, and then washed 3 times with PBS immediately prior to use. In separate experiments, the beads were not washed prior to injection. The coated ~~Affi-Blue~~ AFFI-BLUE agarose beads were coinjected with the 10<sup>7</sup> A549 cells subcutaneously into the flanks of NCJ nude mice. In a separate study 10<sup>7</sup> A549 cells were coinjected with 20 µg of an anti-BMP-2 monoclonal antibody reported to inhibit its activity. As a control, A549 cells were coinjected with 20 µg of an isotope control antibody. Fourteen to 19 days following injection the animals were sacrificed and the tumors were removed and measured in 3 dimensions (length x width x depth). The mice studies were approved by the Robert Wood Johnson Medical School Institutional Animal Care and Use Committee.--

Please replace paragraph 00180 at page 74 with the following paragraph:

-BMP-2 and BMP-4 have been shown to stimulate the migration of non-cancerous human cells. Since migration is important for tumors to invade and metastasize, it was examined whether recombinant BMP-2 stimulates the migration of the A549 and H7249 human lung cancer cell lines *in vitro*. When recombinant BMP-2 was placed in the lower well of a migration chamber, it caused a dose responsive



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increase in the migration of both the A549 and H7249 cells. The BMP-2 antagonist, noggin, completely inhibited the ability of BMP-2 to stimulate the migration of the A549 cells. Next, it was determined whether BMP-2 enhanced migration of the lung cancer cell lines growing in monolayer cell culture. The A549 and H7249 cells were cultured on glass cover slips and placed in 6 well plates containing ~~Affi-Blue~~ AFFI-BLUE agarose beads coated with PBS with 0.1% gelatin or recombinant BMP-2. After 5 days, the number of cells that had migrated off the cover slips and were growing in the six well plate was assessed. There was only an occasional cluster of A549 or H7249 cells growing on the six well plates when cultured with control beads. However, when A549 and H7249 were cultured with ~~Affi-Blue~~ AFFI-BLUE agarose beads coated with recombinant BMP-2, there was consistently a large number of cells that migrated off the cover slips and were growing on the six well plate.--

Please replace paragraph 00183 on page 75 with the following paragraph:

-Since BMP-2 is a secreted protein, it was hypothesized that it may ~~effect~~ affect the growth of the A549 cells differently *in vivo* than it does *in vitro*. To answer this question, required assessment of tumor growth of the A549 cells in athymic nude mice

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treated with recombinant BMP-2 or by inhibiting BMP-2 activity with noggin or anti-BMP-2 antibody. Noggin coated ~~Affi-Blue~~ AFFI-BLUE agarose beads were ~~co-injected~~ coinjected with A549 cells subcutaneously into nude mice. Noggin has a high binding affinity for BMP-2 and BMP-4 preventing their activation of the BMP receptors. Noggin treated 549 cells (n=14) consistently formed tumors which were less than half the size of A549 cells treated with albumin (n=15). ~~Co-injection~~ Coinjection of A549 cells with an anti-BMP-2 monoclonal antibody previously reported to inhibit BMP-2-induced migration of smooth muscle cells<sub>7</sub> (n=4) also produced an approximately 60% reduction in tumor growth when compared to mice ~~co-injected~~ coinjected with a control antibody (n=4).--

Please replace paragraph 00184 at page 76 with the following paragraph:

-A549 cells ~~co-injected~~ coinjected into nude mice with ~~Affi-Blue~~ AFFI-BLUE agarose beads coated with recombinant human BMP-2 (n=15) formed tumors that were approximately 50% larger than that of A549 cells treated with BSA (n=15). Tumors were stained with hematoxylin and eosin and examined by a surgical pathologist for the presence of bone and/or cartilage. There was no evidence of bone and/or cartilage in any of the tumor formed from A549 cells

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treated with recombinant BMP-2. Together these data indicate that BMP-2 produced from the A549 cells enhances tumor growth *in vivo*, which is not associated with the formation of bone.--

Please replace paragraph 00190 at page 79 with the following paragraph:

-To assess the effects of BMP-2 on tumor growth *in vivo* A549 cells were coinjected subcutaneously into female NCJ athymic nude mice with recombinant BMP-2 or the BMP-2 antagonist, noggin. Recombinant protein was delivered to the tumors using ~~Affi-Blue~~ AFFI-BLUE agarose beads as previously described. 25 µg of ~~Affi-Blue~~ AFFI-BLUE agarose beads were coated with 20 µl of 100 µg/ml of albumin, or recombinant human BMP-2. The coated ~~Affi-Blue~~ AFFI-BLUE agarose beads were ~~co-injected~~ coinjected with the 10<sup>7</sup> A549 cells subcutaneously into the flanks of NCJ nude mice. Fourteen to 19 days following injection the animals were sacrificed and the tumors were snapped frozen in liquid nitrogen and protein collected for Western blot analysis. A section of tumor was also placed in ~~Optimal Cutting Temperature (OCT)~~ OCT then frozen for immunohistochemistry studies.--

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Please replace paragraph 00202 at page 84 with the following paragraph:

-1 X107 A549 cells were subcutaneously injected into the flanks of NCR nude mice with ~~Affi-Blue~~ AFFI-BLUE agarose beads (Biorad, Hercules, CA) coated with BSA or recombinant human BMP-2. 25 ug of ~~Affi-Blue~~ AFFI-BLUE agarose were incubated with 2 g of BSA or recombinant BMP-2 for 2 hours and washed with PBS. Tumors were collected 4-6 or 12-14 days later. Tumors were placed in ~~O.C.T.~~ OCT and frozen in liquid nitrogen.--

Please replace cited reference 1 at page 86 with the following:

--1. American Cancer Society, What are the Key Statistics for Lung Cancer. www.cancer.org. 2003. (which can be found at the webpage index at the cancer.org website on the world wide web).--